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Expression and Cellular Distribution Pattern of Plasma Membrane Calcium Pump Isoforms in Rat Pancreatic Islets

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Abstract. This work is aimed at identifying the presence and cellular distribution pattern of plasma membrane calcium pump (PMCA) isoforms in normal rat pancreatic islet. Microsomal fractions of isolated islets and exocrine tissue were analyzed to detect different PMCA isoforms. The cellular distribution pattern of these PMCAs in the islets was also studied in fixed pancreas sections incubated with antibodies against PMCAs and insulin. Antibody 5F10, which reacts with all PMCA variants, showed multiple bands in the blots in the 127–134 kDa region, indicating the presence of several isoforms. Microsomes also reacted positively with specific antibodies for individual PMCA isoforms, generating a band of the expected size. Antibody 5F10 immunocytochemically labeled the plasma cell membrane of both β- and non-β-cells, but predominantly the former. All islet cells were also labeled with antibodies against isoforms 1 and 4, while the antibody reacting with isoform 3 labeled exclusively β-cells. A few β- and non-β-cells were positively labeled with the antibody reacting with PMCA b variant. Negative results were obtained with the antibody against isoform 2. Further studies, together with previous reports on the modulatory effect of insulin secretagogues and blockers upon PMCA activity, may provide evidence of the importance of this particular PMCA expression for islet function under normal and pathological conditions.

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Introduction

An increase in intracellular Ca^{2+} in β-cells is a key signal for the stimulation of insulin secretion by glucose (Wollheim & Sharp, 1981). Although an increased Ca^{2+} flux across the plasma membrane represents the main cause of glucose-mediated insulin secretion, Ca^{2+} mobilization from intracellular stores also participates in this process (Hellman, 1986). As occurs in other tissues, the ensuing elevation in cytosolic Ca^{2+} is rapidly reverted to basal levels by Ca^{2+}-pumps located in the plasma membrane (extrusion), and in the endoplasmic reticulum and mitochondria (uptake) (Carafoli, 1991a).

Several authors have demonstrated the presence of Ca^{2+}-ATPases in rat islets and characterized their properties (Levin, Kasson & Driessen, 1978; Kotagal et al., 1985; Rossi et al., 1988; Gronda, Rossi & Gagliardino, 1988). We have further shown that: (a) Glucose produces a transient and dose-dependent inhibition of plasma membrane Ca^{2+} pump (PMCA), affecting its ATP-dependency; (b) several insulin secretagogues transiently inhibit PMCA activity, while insulin-secretion blockers stimulate it, in a transient manner as well (Gronda et al., 1988; Gagliardino & Rossi, 1994). Thus, we and other authors have suggested that insulin secretagogues or blockers might regulate the islet PMCA either positively or negatively. Such regulation of the islet PMCA activity

might switch either on or off the Ca^{2+} signal for different β-cell functions (Levin et al., 1978; Gronda et al., 1988; Hoenig, Lee & Ferguson, 1990; Gagliardino & Rossi, 1994; Levy, Zhu & Dunbar, 1998).

PMCA is a calmodulin-regulated P-type ATPase that is encoded by a multigene family (Carafoli, 1991a). Mammalian cDNA library screening has indicated the existence of at least four gene products, namely PMCA 1–4 (Stauffer et al., 1993). Isoenzymes 1 and 4 are transcribed in most tissues, whereas isoenzymes 2 and 3 are significantly expressed only in excitable tissues (muscle and brain) (Carafoli & Stauffer, 1994). In addition, alternative RNA splicing can change the structure of its calmodulin-binding domain. These changes affect calmodulin and ATP affinity: E.g., PMCA2 had higher affinity for calmodulin and for ATP than did the PMCA4 expressed in the same system (Enyedi et al., 1994; Hilfiker, Guerini & Carafoli, 1994). Since this shift in Ca^{2+} activation occurs within the Ca^{2+} concentration range crucial for cellular function, it is probable that the alternative splicing has great physiological importance (Enyedi et al., 1994). We have also recently found that different isoforms differ not only in their steady-state but also in their transient kinetic properties (Caride et al., 1999). Thus, identification of the different islet PMCAs might lead to a better understanding of the regulation of Ca^{2+} concentration in islet cells.

The mRNA of different PMCA isoforms in the islets has been identified in intact islets and in several β-cell lines (Varadi, Molnar & Ashcroft, 1995, 1996; Kamagate et al., 2000). The rat exocrine pancreas predominantly transcribes PMCA1 mRNA, whereas β-cells transcribe isoforms 1 to 4 (Kamagate et al., 2000) or 1, 2 and 4 (Varadi et al., 1995, 1996), as well as six alternative variants at splice sites A, B, and C (Varadi et al., 1995, 1996; Kamagate et al., 2000). The presence of their corresponding proteins was studied using either antibody 5F10 — which reacts with all PMCA isoforms (Varadi et al., 1995, 1996) — or antibodies that specifically react with each one of the isoforms (Kamagate et al., 2000). Because the amount of mRNA sometimes does not correlate with the amount of protein (Devarajan, Gilmore-Hebert & Benz, 1992), further measurement of the PMCA-protein expression with available specific antibodies is important. Therefore, we (a), screened rat pancreatic islet microsomes with specific antibodies against the different PMCA isoforms and (b), further studied their cellular expression pattern in normal rat islet cells.

Materials and Methods

Islet Isolation

Pancreatic islets from adult male Wistar rats were isolated by collagenase (SERVA, Heidelberg, Germany) digestion (Lacy & Kostianovsky, 1967). Isolated islets as well as samples of exocrine-digested tissue were collected and fixed (*see* below) to perform morphological studies.

Preparation of Islet- and Exocrine Tissue- Microsomal Fractions

Islet Microsomal Fraction

The isolated islets were collected and washed in phosphate-buffered saline (PBS) containing 1 mM EDTA. After equilibration for 15 min in hypotonic solution of 10 mM Tris/HCl, pH 7.5, containing (in mM) 1 $MgCl_2$, 0.1 PMSF, 2 dithiothreitol, 1 EDTA, and 4 μg/ml aprotinin, the tissue was homogenized. An equal volume of 10 mM Tris/HCl, pH 7.5, containing 0.3 M KCl, 0.5 M sucrose, 2 mM dithiothreitol and 1 mM EDTA was added to the homogenates, and the mixture was further homogenized. Unbroken cells, tissue debris and nuclei were removed by centrifugation at 4,000 × g for 15 min at 4°C. The supernatant was finally centrifuged at 100,000 × g for 90 min at 4°C, and the pellet resuspended in (in mM) 10 Tris/HCl, pH 7.2, 120 NaCl, 2 dithiothreitol, 2 EDTA, 0.1 PMSF, 0.1 benzamidine, 8 μg/ml aprotinin. The microsomal fraction was kept in liquid nitrogen until further use.

Exocrine Tissue Microsomal Fraction

Following islet isolation by collagenase digestion, the remaining pancreatic tissue was used to obtain this fraction. This material was treated using the same procedure as that described to obtain the islet microsomal fraction.

SDS-PAGE

This procedure was performed in vertical discontinuous slab gels. Twenty microliters of islet — or exocrine tissue — membrane samples (equivalent to 1,000 islets or the corresponding amount of protein [500 mg] from exocrine tissue, respectively) were placed into the wells of the stacking gel containing 4% acrylamide, 125 mM Tris/HCl, pH 6.8, 0.1% SDS, 0.05% ammonium persulfate, and 0.2% TEMED. The running gel composition was 7.5% acrylamide, 375 mM Tris-HCl, pH 8.8, 0.1% SDS, 0.2% ammonium persulfate, and 0.2% TEMED. The reservoir buffer contained 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. The gel slabs (10 × 10 cm, 0.5 mm thickness) were placed at room temperature and samples run at 60 V until they entered the gel; then the voltage was increased up to 100 V until the front was about 0.5 cm from the bottom.

Western Blotting and Immunostaining

The slabs were then electroblotted onto a poly (vinylidene difluoride) microporous membrane (Immobilon) by means of a discontinuous buffer system. Before immunostaining, the Immobilon sheets were treated with blocking solution and then incubated with appropriate dilutions of the primary antibodies (*see* below) in PBS at room temperature for 1 hr. Saturating antibody concentrations were used for all the immunostaining. After rinsing with PBS, the streptavidin-phosphatase conjugate anti-mouse and -rabbit IgG (Sigma, St Louis, MO) was added for 1 hr; this step was followed by rinsing. Color development was obtained using 3 amino-9-ethylcarbazole (Sigma).

The density of the bands was measured using a Kodak DC290 digital camera and the Kodak 1 D Image Analysis Software.

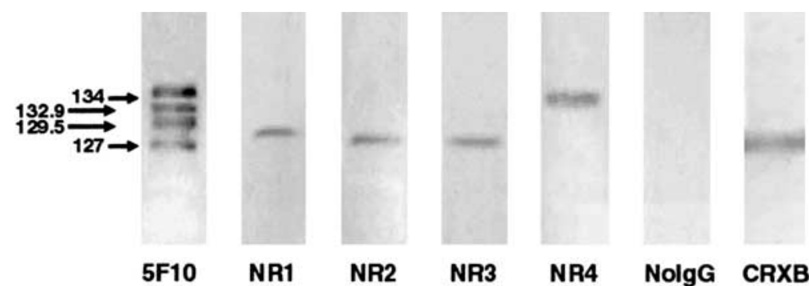


Fig. 1. Western blot of islet microsomal fractions performed with specific antibodies against the different PMCA isoforms (*see Material and Methods*). The antibody employed is shown below each pair of lanes. Lane 6 indicates the negative control (omission of the first specific antibody) for islet microsomal fractions.

Antibodies

The monoclonal antibodies used here were raised against the human erythrocyte Ca^{2+} pump. Specificity was demonstrated by their reaction with the purified enzyme in an ELISA system and by their staining in a Western blot (Filoteo et al, 1997; Caride et al., 1996; Adamo, Caride & Penniston, 1992). The individual antibody specificity is as follows: monoclonal antibody 5F10 for all the PMCA isoforms; the remaining antibodies are polyclonal, and their specificity is as follows: NR1 for rat(r) and human(h) PMCA1; NR2 for r and h PMCA2; NR3 for r and h PMCA3; NR4 for r and h PMCA4; CRXB for b, c, and d forms of rPMCAl, 2, and 3.

Morphological Studies

Samples of the pancreas tail were fixed in cold 10% formaldehyde and embedded in paraffin. Sections thinner than 5 μm were obtained from different levels of the block; they were mounted on slides and stained for the immunocytochemical identification of PMCAs and β-cells.

Identification of Islet PMCAs and β-Cells

Deparaffinized sections were pretreated with 250 ml of antigen-retrieval solution (Vector Lab., Burlingame, CA, USA) for 10 min in a microwave oven (500 W). The sections were then treated with normal porcine serum to prevent nonspecific staining, and endogenous peroxidase was blocked with methanol/hydrogen peroxide; thereafter, the sections were sequentially incubated with appropriate dilutions of the previously described PMCA antibodies and our own guinea-pig anti-insulin serum (1:20,000).

Double-Immunolabeling Studies

Double staining of the following pairs was performed: PMCA (PMCA antibodies) and β-cells (insulin antibody). We used the streptavidin-biotin complex method, with peroxidase (PMCAs) and alkaline phosphatase (β-cells) together with carbazole and fast-blue, respectively, as chromogens. Incubations with the primary antibodies were overnight, whereas those with the secondary biotinylated antibodies were for 30 min. We verified serologic specificity by omitting the first peptide-specific antibody in the immunostaining procedure.

Results

The immunoblot analysis of normal rat islet microsomes using specific antibodies against PMCA isoforms is shown in Fig. 1. No bands were observed when the first specific antibody was omitted (lane 6). Conversely, major polypeptide bands reacting with antibody 5F10 (lane 1) were in the molecular mass range of 127–134 kDa, which is the expected range for PMCA (Filoteo et al., 1997; Caride et al., 1996; Adamo et al., 1992; Carafoli, 1991b). The presence of several bands suggests that more than one variant of the pump is present. However, the possible existence of proteolytic and aggregation products should also be considered.

A Western blot of the islet microsomal fractions was also performed using saturating concentrations of antibodies NR1, NR2, NR3 and NR4, which specifically react with PMCA isoforms 1, 2, 3 and 4, respectively (Fig. 1, lanes 2 to 5). Islet microsomal fractions showed single positive bands when tested against all these antibodies, as has been reported in brain microsomes (Filoteo et al., 1997).

Microsomes also showed a band with similar characteristics when incubated with the CRXB antibody, which reacts with the b variant of PMCAs 1, 2 and 3 (Fig. 1, lane 7). The size of this band suggests that variants 2b or 3b (or both) are present in pancreatic islets. The size of the band stained with antibody NR1 is too small for the expected size of PMCA1b (molecular mass = 134,715). Therefore, the variant of PMCA1 present might be either 1a (molecular mass = 128,919), or a proteolytic product of PMCA1b.

The immunoblot analysis of digested exocrine tissue microsomes showed a single band in the molecular mass range expected for PMCA, when incubated with antibody 5F10 (Fig. 2*c*). The density of this band — measured by densitometric analysis — was at least two times lower than that observed using the same antibody and an equivalent amount of islet microsome protein (Fig. 2*e*). This band was not present when the first specific antibody was omitted (*not shown*).

Figure 3 shows representative paired pictures of islet sections simultaneously stained with different PMCAs (left panel, peroxidase staining) and insulin antibodies (right panel, phosphatase staining). It can

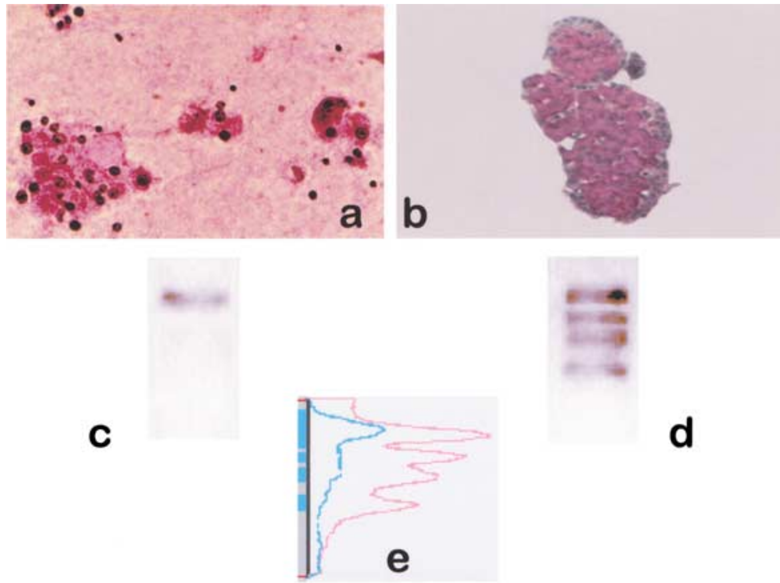


Fig. 2. (*a*) Smear of exocrine digested tissue stained with hematoxylin-eosin. X 20. (*b*): Islet isolated (collagenase digestion, fixed in 10% *p*-formaldehyde) and embedded in paraffin. A thin section was stained with hematoxylin and with insulin antibody-peroxidase and revealed with 3 amino-9-ethylcarbazole. × 20. (*c* and *d*): Western blot of microsomes from exocrine digested tissue and isolated islets (similar amount of total protein), respectively, incubated with antibody 5F10. While islet microsomes showed several bands corresponding to different PMCA isoforms, a single band appeared when microsomes of the exocrine tissue were incubated with the antibody. (*e*): Densitometric measurement using a Kodak DC290 digital camera and the Kodak 1D Image Analysis Software. Values from islet bands (red) were twice higher than those from exocrine tissue (blue).

be seen that in the islets, antibody 5F10 labeled the plasma cell membrane of both β- and non-β-cells (Fig. 3*a* and *b*), but predominantly the former. All islet cells were also labeled with antibodies against isoforms 1 and 4 (Fig. 3*c* and *d*, *g* and *h*, respectively), while the antibody reacting with isoform 3 labeled exclusively β-cells (Fig. 3*e* and *f*). A few β- (located at the external part of the islets) and non-β-cells were positively labeled with antibody CRXB, which reacts with PMCA b variant (Fig. 4*a* and *b*). Negative results were obtained with the antibody against isoform 2 (*not shown*).

Discussion

Western blots of normal rat pancreatic islet homogenates were performed with specific antibodies against the different PMCA isoforms (Adamo et al., 1992; Caride et al., 1996; Filoteo et al., 1997), revealing protein bands compatible with the Ca^{2+} pump characteristics (Carafoli, 1991b; Adamo et al., 1992; Caride et al., 1996; Filoteo et al., 1997). Furthermore, the total area under the curve in the densitometric scans shown in Fig. 2 was more than 10-fold larger in the islets than in the exocrine pancreas. This is consistent with the lack of staining in the histological samples of exocrine tissue, as compared to the islets, thus suggesting that the islets are particularly rich in PMCAs.

Islet microsomes reacted positively with all the isoform-specific antibodies tested, giving a single band at a kDa range close to the expected molecular weight of the PMCAs, suggesting that all these antibodies are reacting with the Ca^{2+} pump. No marked differences were observed in band intensity among the different antibodies used. Since the width and intensity of the bands depend on both the amount of reacting protein and the antibody affinity, no quantitative conclusions can be drawn about the relative amount of each PMCA isoform in the islets.

An antibody that reacts with variant b of PMCAs 1, 2 and 3 gave also a strong band within the expected size.

From the results we can infer that normal rat islets express the spliced variant of all four PMCAs, and probably the b variant of isoforms 1, 2 and 3. Regarding the presence of PMCA3, while Kamagate et al. (2000) and we have shown its presence in rat islets, other authors have reported negative results (Varadi et al., 1995, 1996). Thus, these results demonstrate that all the different isoforms and spliced variants identified in the islets by RT-PCR (Varadi et al., 1995, 1996; Kamagate et al., 2000) are expressed in normal rat islets. The fact that so many variants are expressed in this tissue suggests that these pumps are important for the regulation of calcium signaling, an essential feature for insulin secretion.

The presence of isoenzymes 1 and 4 confirmed the fact that they are transcribed in most tissues,

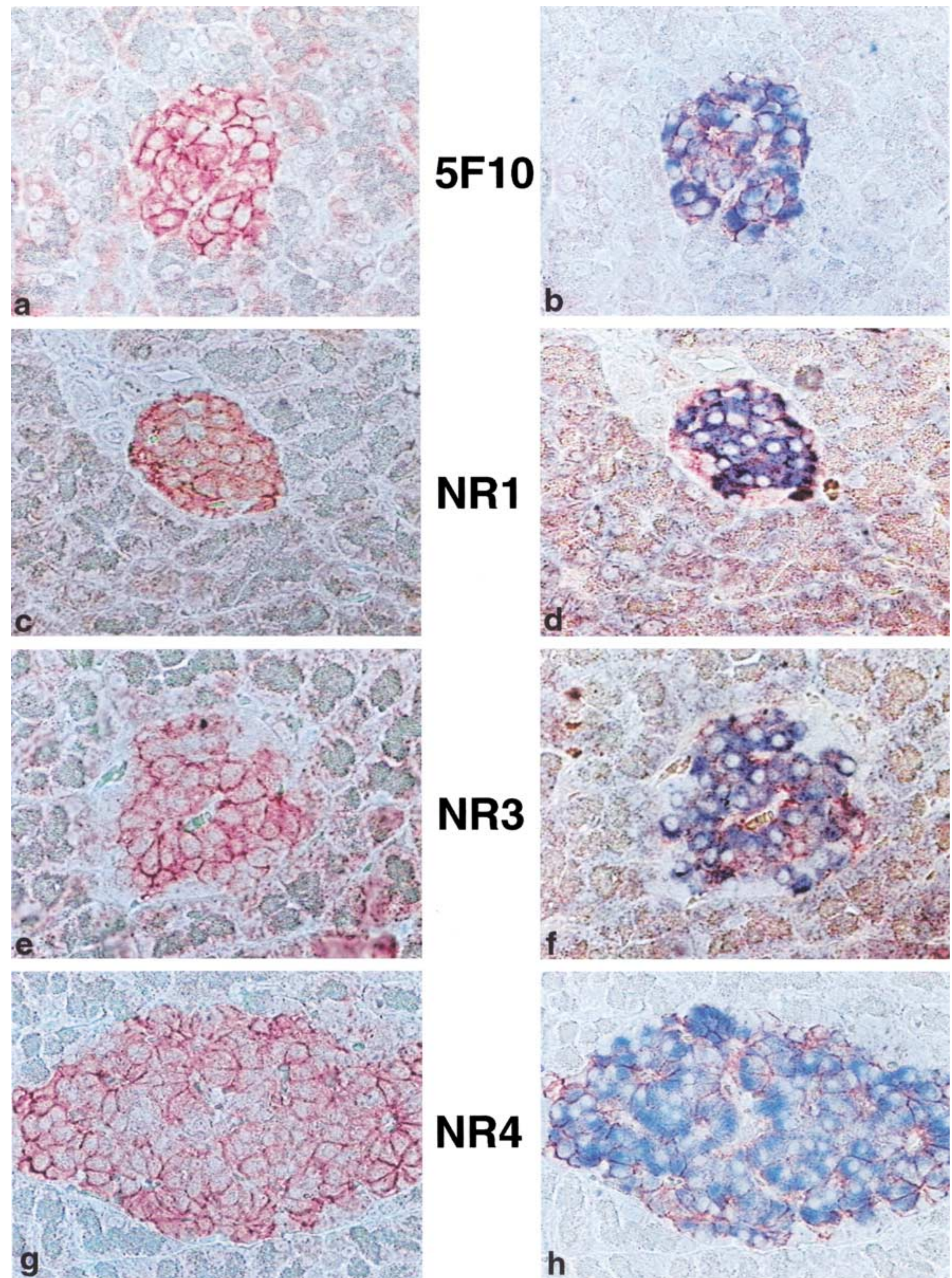
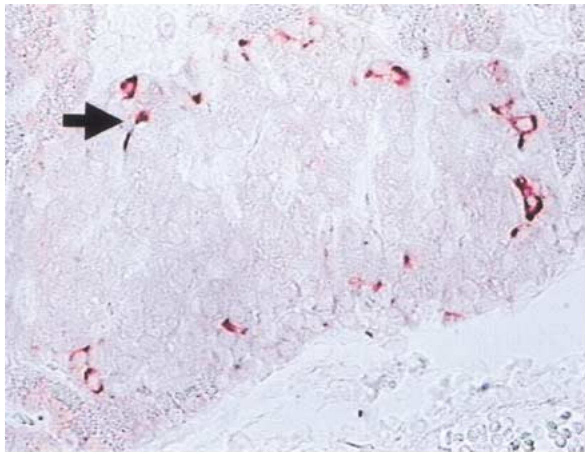


Fig. 3. Immunocytochemical demonstration of PMCA isoforms in normal rat islet cells. Left panel: Presence of PMCA revealed by peroxidase (red). Right panel: The same islet sections stained with insulin antibodies and revealed by phosphatase (blue). The PMCA antibodies used are shown in the middle of every pair of pictures. (*a* and *b*): Comparing both panels, it can be seen that the 5F10 antibody labeled the plasma membrane of most islet cells (β- and non-β-cells). (*c* and *d*): The NR1 antibody labeled most islet cells, even though with a greater intensity those located in the islet mantle (non-β-cell area). (*e* and *f*): Antibody NR3 labeled exclusively the plasma membrane of β-cells. (*g* and *h*): Antibody NR4 labeled both β- and non-β-cells. A stronger staining was seen in β-cells located at the outer portion of the islet × 200.



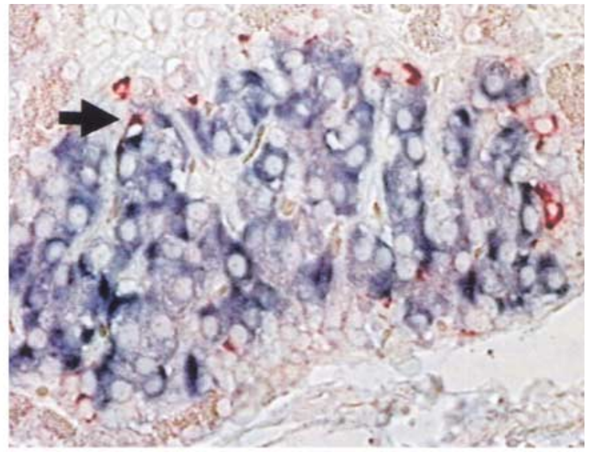


Fig. 4. Immunocytochemical demonstration of the PMCA b isoforms with CRXB in normal rat islet cells. Left panel: b-isoforms revealed by peroxidase. Right panel: The same islet section, stained with the insulin antibody and revealed by phosphatase. CRXB-stained β- (arrows), and non-β-cells.

while isoenzymes 2 and 3, which are significantly expressed only in excitable tissues, namely muscle and brain (Carafoli & Stauffer, 1994), and inner ear (Dumont et al., 2001), are also present in normal rat islet cells.

The immunocytochemical studies showed that PMCAs 1, 3, and 4 were identified in endocrine cells with a distinctive distribution pattern. The selective presence of PMCA3 in β-cells is remarkable and consistent with the mRNA pattern recently reported by Kamagate et al. (2000). It is known that PMCA3 is expressed in cells with special functions: PMCA3f was identified in skeletal muscle (Filoteo et al., 2000), while other PMCA3 variants are located in specific structures of the brain (Filoteo et al., 1997), the inner ear (Dumont et al., 2001), and a small amount is in the thin descending limb of the Henle loop in the kidney (Caride et al., 1995). Thus, its presence in β-cells is consistent with this restrictive and selected expression pattern, also suggesting that this isoform is perhaps the main one responsible for the changes measured in islet PMCA activity in response to insulin secretagogues (Gronda et al., 1988; Gagliardino & Rossi, 1994; Hoenig et al., 1990; Levy et al., 1998).

The antibody that reacts with the b variant of PMCAs 1, 2 and 3 also labelled islet cells.

We do not have at the moment a plausible explanation for the immunocytochemically negative results obtained with PMCA2, even though we found this isoform by Western blot analysis of islet microsomes. However, it might be argued that the cellular concentration of this isoform is lower than the others, being below the sensitivity of our immunocytochemical assay procedure.

In immunocytochemical experiments, exocrine cells were not labeled with any of the PMCA antibodies at the dilutions currently employed. This differential reactivity could be ascribed to the apparent lower concentration of PMCA-protein we have measured in exocrine cells (Fig. 3).

In summary, our results demonstrate the protein expression of PMCAs 1 to 4 and some spliced variants in normal rat islets, providing further support to previous studies mainly based on mRNA measurements (Varadi et al., 1995, 1996; Kamagate et al., 2000). They also show for the first time the immunocytochemical distribution pattern of PMCA in islet cells, with a selective location of PMCA3 in β-cells, which is unusual in cells other than skeletal muscle and neuronal tissue (Carafoli & Stauffer, 1994; Filoteo et al., 1997, 2000). Further studies, together with previous reports on the modulatory effect of insulin secretagogues and blockers upon PMCA activity, may provide evidence of the importance of this particular PMCA expression for islet function under normal and pathological conditions.

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